Indole-Based Compounds as a New Class of Allosteric Inhibitors of HIV-1 Integrase

Research Thesis

Presented in partial fulfillment of the requirements for graduation
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by

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Abstract

HIV-1 integrase (IN) is an important therapeutic target in the fight against AIDS as its function is essential for viral replication. Constant mutations in the HIV-1 genome allow the virus to gain resistance to current antiretroviral therapies. Therefore, there is a continued need to discover new therapeutic compounds with alternative mechanisms of action. One such mechanism is to allosterically inactivate IN through a small molecule inhibitor promoting aberrant IN multimerization. IN functions as a tetramer, and if it is multimerized further by a compound then IN will no longer be able to carry out its function properly. Another mechanism is to interfere with HIV-1 IN binding to its principal cellular interacting partner lens epithelium-derived growth factor (LEDGF)/p75. The aim of this study was to utilize novel homogeneous time-resolved fluorescence (HTRF)-based assays to identify promising new lead compounds and dissect their mechanism of action. Six indole-based compounds were initially analyzed by HTRF-based LEDGF/p75-dependent integration assay and compound KF113 with an IC_{50} value of ~ 4.5 μM was selected for further structure-activity relationships studies. KF113 exhibited a multimodal mechanism of action as this compound inhibited IN binding to LEDGF/p75 as well as induced aberrant IN multimerization. Importantly, KF113 was fully active against mutant A128T IN, which confers marked resistance to current quinoline-based allosteric IN inhibitors. These results indicate that our innovative HTRF-based assays allow for efficient identification of promising inhibitors of HIV-1 IN. Furthermore, the indole-based compounds are promising leads for further development and could ultimately lead to the discovery of novel anti-HIV-1 drugs.

Introduction

Each year in the United States, about 50,000 people are infected with human immunodeficiency virus type 1 (HIV-1). In 2012, 1.2 million people were living with HIV-1 in the United States (*1*). In order to halt the progression of HIV to AIDS, highly active antiretroviral therapy (HAART) has been used over the past 20 years, and has allowed transformation of this once deadly disease into a chronic infection. These drugs are grouped into six different classes, including non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs), fusion inhibitors, CCR5 antagonists (CCR5s), and integrase strand transfer inhibitors (INSTIs). There are currently over thirty FDA-approved antiretroviral medicines to treat HIV-1 infection, with some of these being combination therapies of two or more of the aforementioned drug classes. The current regimen per recommendation of the U.S. Department of Health and Human Services (HHS) often includes two NRTIs in combination with an INSTI. Although these current therapies are effective in reducing viral loads to undetectable levels within weeks of administration, there remains a large number (1 in 8 people, or 12.8% of those infected) of individuals who do not know they are infected, and an even larger percentage of people remain untreated. These individuals may allow for increased transmission of HIV-1. Furthermore, treated individuals continue to have a lower life expectancy than those that are uninfected (*2*). Taking these factors into consideration, as well as the fact that constant mutations in the HIV-1 genome allow the virus to gain resistance to current antiretroviral therapies, there is a continued need to discover new therapeutic compounds with alternative mechanisms of action.

HIV-1 integrase (IN) is a multifunctional virally encoded enzyme that catalyzes the integration of viral DNA copy of the HIV-1 genome into the human chromosome (Figure 1). IN does this in two steps: (1) 3'-processing activity which removes two nucleotides from the 3'-terminus of each end of viral DNA and (2) the strand transfer of the processed viral DNA into the host genome. IN is an important therapeutic target in the fight against AIDS as its function is essential for viral replication. The current FDA-approved drugs that target IN include INSTIs such as raltegravir, elvitegravir and dolutegravir (*3-5*). These drugs have high affinity for the HIV-1 IN active site. However, mutations of IN in the binding sites of INSTIs have been observed in patients, leading to HIV-1 resistance. Therefore, there is a need to research alternative mechanisms of HIV-1 inhibition with compounds that utilize allosteric-binding sites.

Such mechanisms include modulating protein-protein interactions essential for HIV-1 integration. Accordingly, we are interested in identifying new compounds that would stabilize functionally compromised IN multimers. IN functions as a tetramer, so we are interested in compounds that promote aberrant, higher order (greater than the functionally required tetramer) multimerization of IN. Another mechanism is to interfere with HIV-1 IN binding to its key cellular cofactor LEDGF/p75. This human-chromatin associated protein binds the catalytic core domain (CCD) of the IN tetramer and guides the protein to highly active genes in the host genome for viral DNA integration. Compounds that can interfere with the binding of these two proteins are expected to impair HIV-1 replication (*6*). To identify such inhibitors we have developed innovative homogenous time resolved fluorescence (HTRF)-based methodologies, which allow for efficient identification of both types of inhibitors capable of inducing aberrant IN multimerization and/or inhibiting IN-LEDGF/p75 binding (*7*). These improved assays were used to characterize a mode of action of recently discovered quinolone-based allosteric IN inhibitors (ALLINIs), which are currently undergoing clinical trials (*8-10*). However, it was also reported that quinolone-based ALLINIs exert a low genetic pressure for developing resistant strains (6*, 9*). For example, the A128T substitution in the IN coding region readily emerges in cell culture in the presence of quinoline-based ALLINIs. Furthermore, the mutant virus replicates at the wild type levels and confers marked resistance to quinolone-based inhibitors (*9*). Therefore, there is a need to develop improved ALLINIs to overcome this problem. In the present study we have investigated a new class of indole-based derivatives that potently inhibit both the wild type and A128T INs. Therefore, the indole-based compounds synthesized by Dr. Fuchs' group provide promising leads for further development.

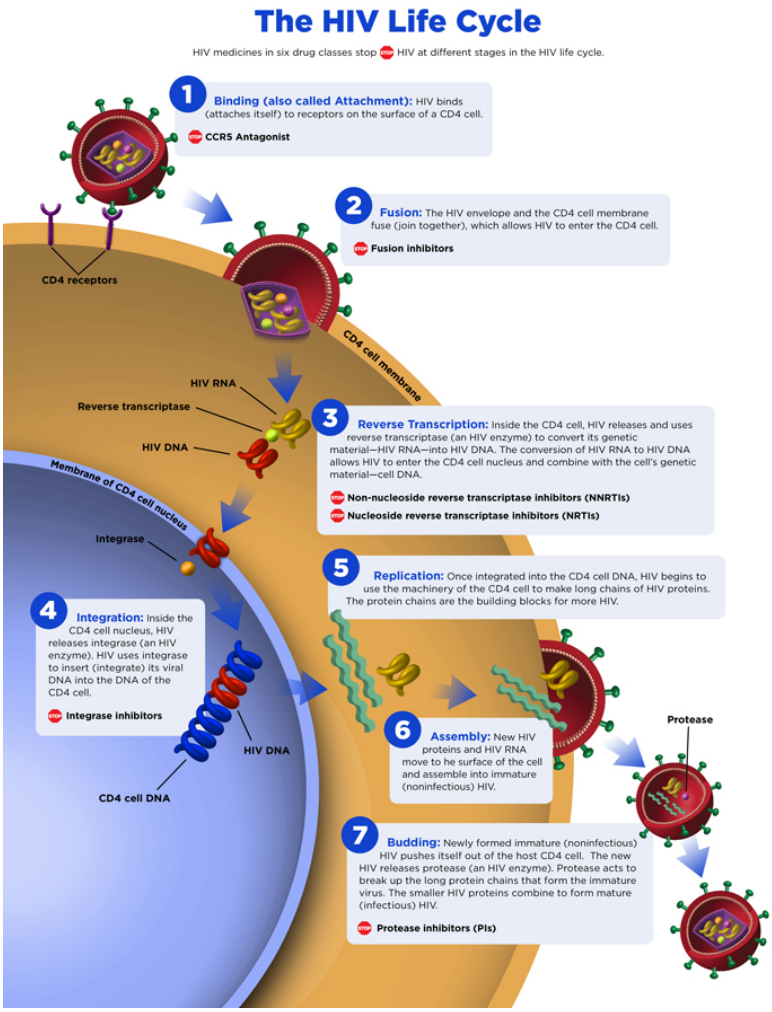


Figure 1. Schematic for HIV-1 replication cycle provided by aidsinfo.nih.gov.

Methods

Homogeneous time-resolved fluorescence (HTRF)-based assays

The HTRF technique utilizes fluorescence resonance energy transfer (FRET) to measure the time-delayed transfer of energy between two fluorophores, a donor (DON) and an acceptor (ACE), when in close proximity (Figure 2). A time delay between the system excitation and fluorescence measurement allows the signal to be cleared of all non-specific short-lived emissions, which in turn enables dissection of true interactions from non-specific fluorescence. The application of this approach can be extended to measure a variety of protein-protein and protein-nucleic acid interactions, where interacting partners are labeled with DON and ACE fluorophores. Therefore, we adapted this technique to monitor IN catalytic activities in the presence and absence of its cognate cofactor LEDGF/p75 as well as to evaluate inhibitory potencies of the compounds of interest. Furthermore, we have extended the application of the

HTRF-based approach to elucidate the ability of small compounds to interfere with IN binding to its cognate co-factor LEDGF/p75 and/or induce IN multimerization.

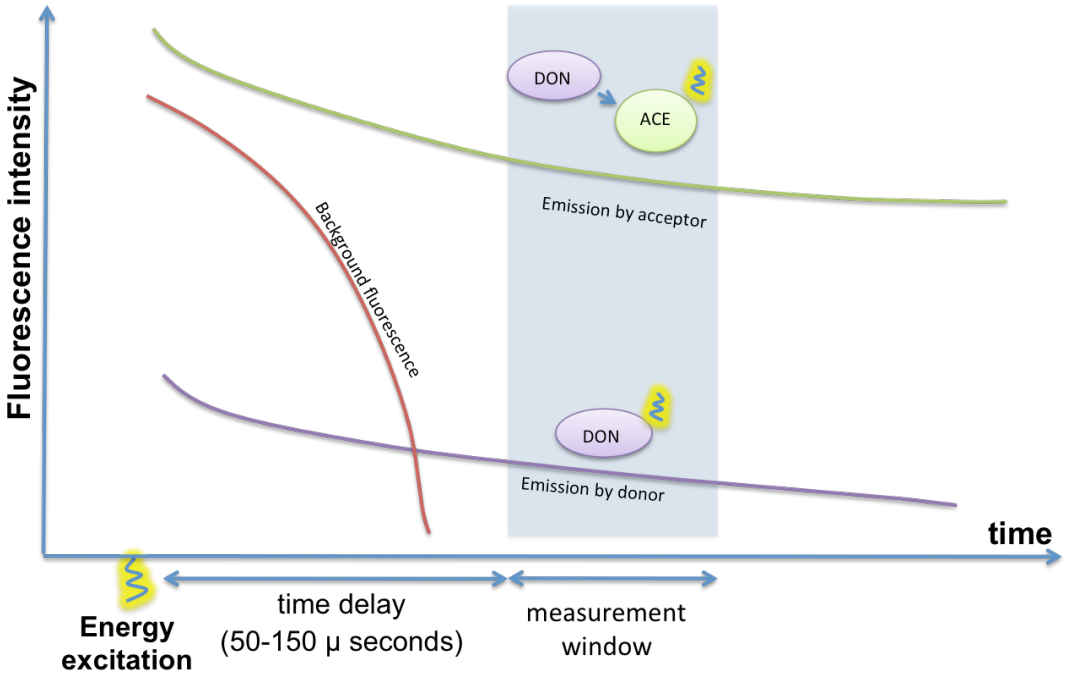


Figure 2. Schematic for HTRF-based assays.

HTRF-based LEDGF/p75-dependent integration assay

This assay measures the IN-catalyzed integration of viral DNA into the target DNA in the presence of LEDGF/p75 (Figures 3, 4 and Table 1). The viral DNA is tagged with Cy5 fluorophore, and the target DNA is biotinylated, allowing it to interact with streptavidin-europium (SA-EU fluorophore). The integration product is monitored by the intensity of a time-resolved signal between these two fluorophores. If the compound inhibits any mechanism of integration, then there is a corresponding decrease in HTRF signal compared to the control signal which is IN that was incubated with diluent DMSO.

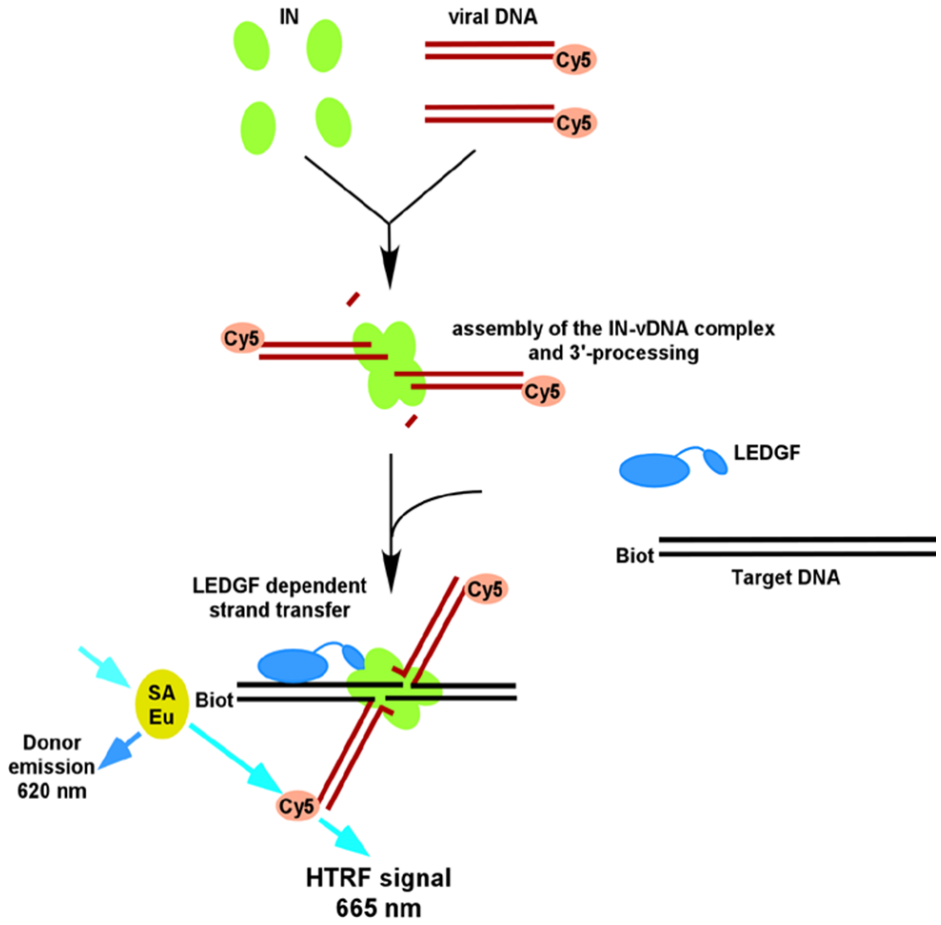


Figure 3. Schematic for LEDGF/p75-dependent integration assay.

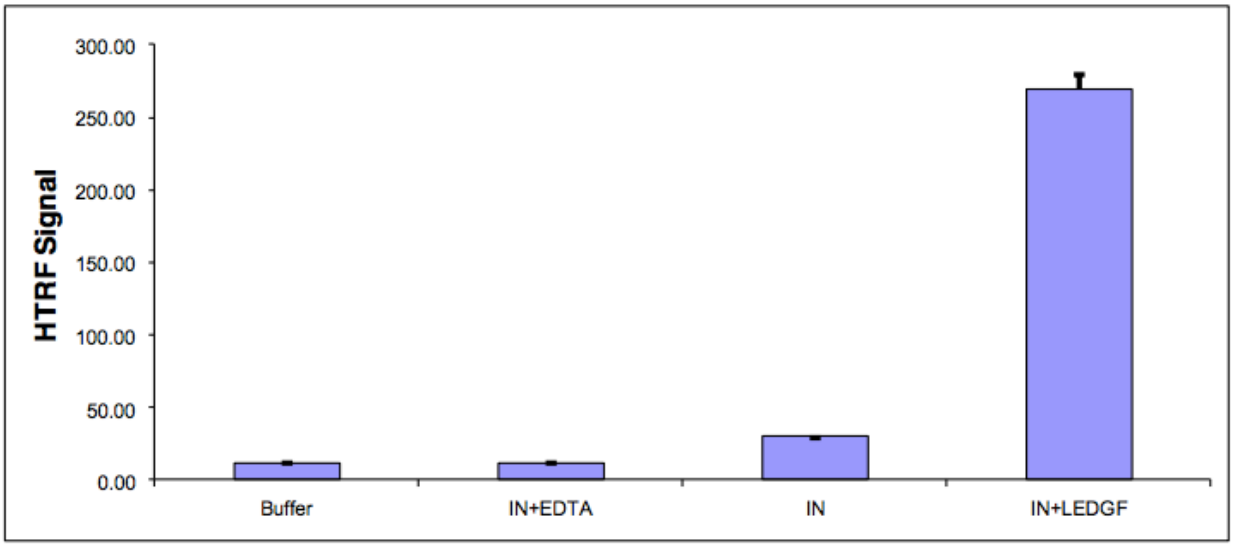


Figure 4. Activity enhancement of DNA integration by LEDGF/p75. IN is an Mg^{2+}-dependent enzyme. Therefore, EDTA, which chelates Mg^{2+} ions, inhibits the reaction yielding the HTRF signal comparable with the buffer background.

Table 1: The protocol for the LEDGF/p75 Dependent Integration assay

	Parameter	Value	Description
1	IN Mix	2.5 µl	Add to 384-well plate
2	Compound or DMSO or BI-B2 (positive control)	20 nl	Tip transfer and mix
3	Incubation time	30 min.	Room temperature. Avoid direct light.
4	DNA substrates + LEDGF/p75	7.5 µl	Tip transfer and mix. Avoid direct light.
5	Incubation time	2.5 hr	37°C. Avoid direct light.
6	Streptavidin-EuCryptate Lance	10 µl	Tip transfer and mix. Avoid direct light.
7	Incubation time	2.5 hr	Room temperature. Avoid direct light.
8	HTRF readout	HTRF ratio	HTRF signal / Donor Emission

HTRF-based LEDGF/p75-independent 3'-processing assay

This assay measures the ability of tested compounds to inhibit the 3'-processing catalytic activity of IN in the absence of LEDGF/p75 (Figure 5 and Table 2). The viral DNA is tagged with Cy5 fluorophore on one 3' end, which is cleaved by IN during the 3'-processing reaction. The 3' end of the complementary DNA stand is biotinylated, allowing it to interact with streptavidin-europium (SA-EU fluorophore). The 3'-processing reaction is monitored by the change of the intensity of a time-resolved signal between these two fluorophores. If IN carries out its catalytic function properly, then one of the fluorophores will be cleaved off, resulting in corresponding decrease of the HTRF signal. However, if the tested compound inhibits IN 3'-processing activity, then the signal remains unchanged.

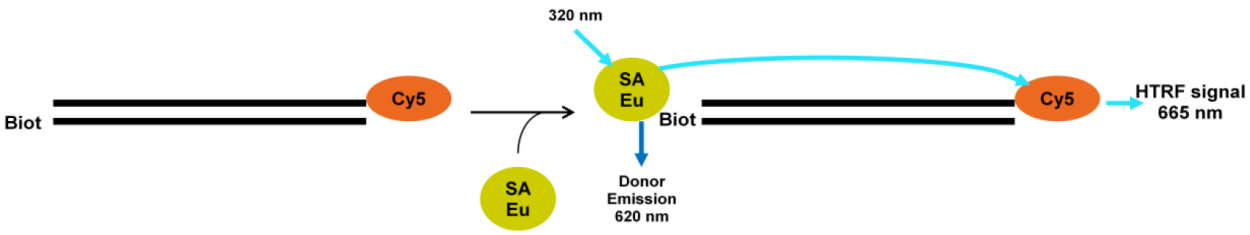


Figure 5. Schematic for the 3'-processing assay

Table 2. The protocol for the 3'-processing assay

	Parameter	Value	Description
1	IN Mix	2.5 µl	Add to 384-well plate
2	Compound or DMSO or BI-B2 (positive control)	20 nl	Tip transfer and mix
3	Incubation time	60 min.	Room temperature. Avoid direct light.
4	DNA substrates	5 µl	Tip transfer and mix. Avoid direct light.
5	Incubation time	2.5 hr	37°C. Avoid direct light.
6	Streptavidin-EuCryptate Lance	10 µl	Tip transfer and mix. Avoid direct light.
7	Incubation time	2.5 hr	Room temperature. Avoid direct light.
8	HTRF readout	HTRF ratio	$\frac{\text{HTRF signal}}{\text{Donor Emission}}$

HTRF-based IN multimerization assay

This assay monitors the interaction between two HIV-1 IN proteins: one labeled with an N-terminal hexa-histidine (6xHis) tag and another labeled with a C-terminal FLAG tag (Figures 6, 7 and Table 3). Two fluorescent antibodies, anti-6xHis-XL665 and anti-FLAG-EuCryptate (Eu), bind to each respective IN tag, allowing fluorescence energy transfer (HTRF signal) upon IN-IN interaction. A higher signal indicates that the mixture contains higher order inactive IN multimers. If the compound stabilizes functionally compromised IN multimers, then there is a corresponding increase in HTRF signal as compared to the background signal of IN that was incubated with diluent DMSO.

The compounds that display activity in the multimerization assay are titrated and run through a counterscreen assay, to eliminate false positive hits. Some compounds exhibit long-lived fluorescence upon energy excitation, leading to an increase in the HTRF signal from a source other than the accepter emission. The compounds that exhibit an increase in the HTRF signal in the absence of the donor fluorophore are eliminated. The counterscreen is run with the same parameters as multimerization assay (Table 3), except that Flag-IN is entirely omitted.

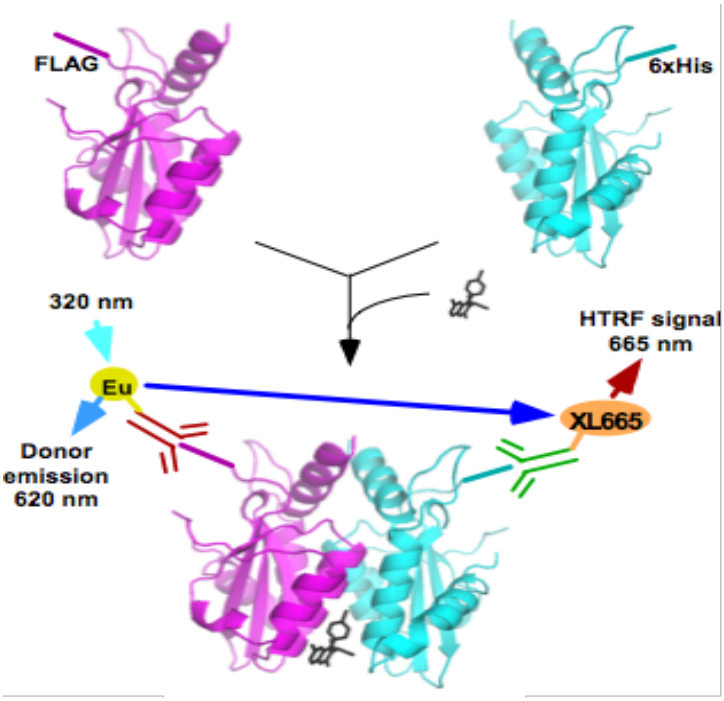


Figure 6. Schematic for HTRF-based IN multimerization assay.

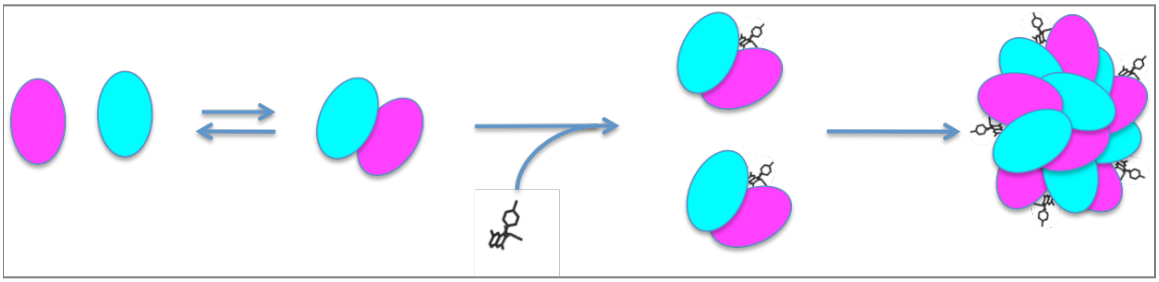


Figure 7. Schematic to show aberrant IN multimerization induced by ALLINIs.

Table 3: The protocol for the IN multimerization assay

	Parameter	Value	Description
1	Flag-IN & His-IN Mix	14 µl	Add to 384-well plate
2	Compound or DMSO or BI-B2 (positive control)	40 nl	Tip transfer and mix
3	Incubation time	3 hr	Room temperature
4	Antibody Mix	6 µl	Tip transfer and mix
5	Incubation time	2.5 hr	Room temperature. Avoid direct light.
6	HTRF readout	HTRF ratio	HTRF signal / Donor Emission

HTRF-based IN-LEDGF/p75 binding assay

This assay monitors the interaction between HIV-1 IN and LEDGF/p75. HIV-1 IN is labeled with an N-terminal hexa-histidine (6xHis) tag and LEDGF/p75 is labeled with a C-terminal FLAG tag (Figure 8 and Table 4). Two fluorescent antibodies, anti-6xHis-XL665 and anti-FLAG-EuCryptate (Eu), bind to each respective tag, allowing fluorescence energy transfer (HTRF signal) upon IN-LEDGF interaction. Prospective inhibitors of IN binding to LEDGF/p75 will lead to a decrease in fluorescent signal in comparison to diluent DMSO.

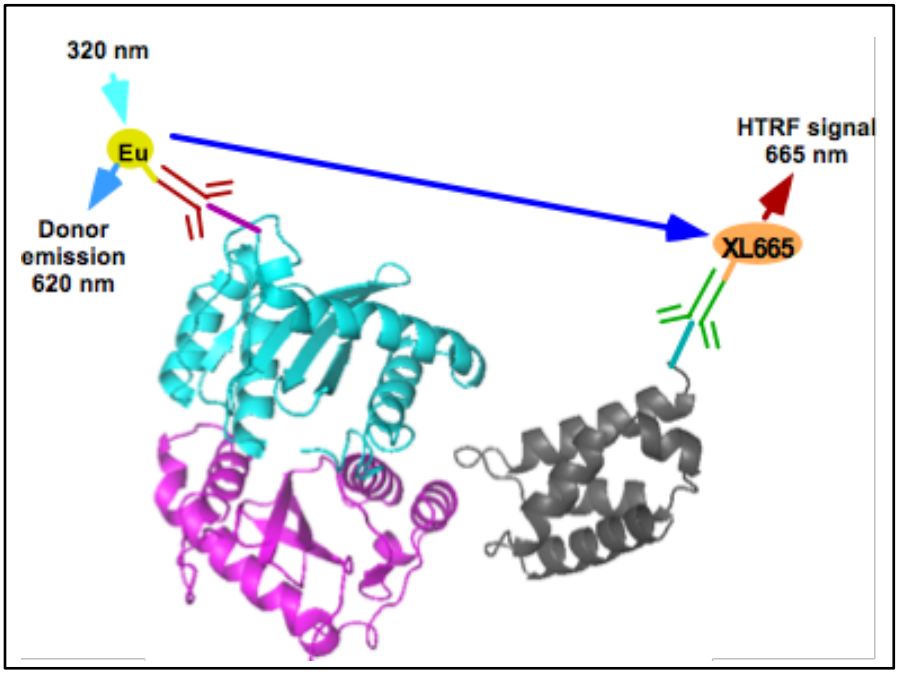


Figure 8. Schematic for the HTRF-based IN-LEDGF/p75 binding assay.

Table 4: The protocol for the IN-LEDGF/p75 binding assay.

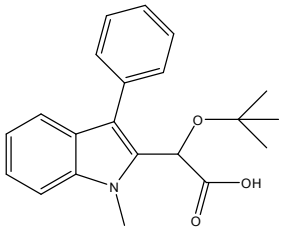
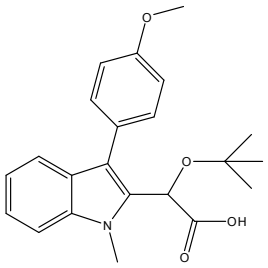
	Parameter	Value	Description
1	His-IN	25 µl	Add to 384-well plate
2	Compound or DMSO or BI-B2 (positive control)	2 µl	Tip transfer and mix
3	Incubation time	1 hr	Room temperature
4	LEDGF/p75-Ab Mix	6 µl	Tip transfer and mix
5	Incubation time	3 hr	4°C. Avoid direct light.
6	HTRF readout	HTRF ratio	HTRF signal / Donor Emission

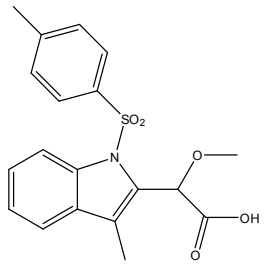
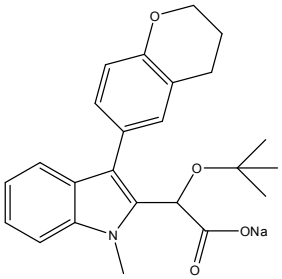
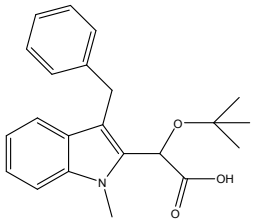
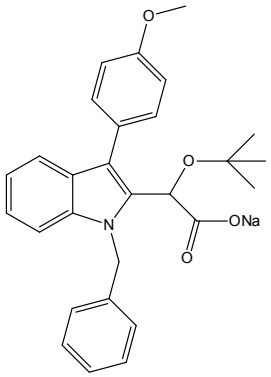
Results

Activities and the mode of action of indole-based compounds.

I have analyzed six indole-based compounds (Table 5) synthesized by Dr. Fuchs' laboratory. For initial experiments I have used the LEDGF/p75-dependent integration assay because this method can capture IN inhibitors with different modes of action. For example, compounds that impair either IN catalytic activity, induce aberrant IN multimerization or inhibit IN-LEDGF/p75 binding all can be identified by this approach. Comparative analyses of IC_{50} value for the six indole-based compounds have revealed KF113 as the most promising lead with an IC_{50} value of ~ 4.5 μM (Table 5).

Table 5. Chemical structures and respective IC_{50} values of indole-bases compounds evaluated in LEDGF/p75-dependent integration assays.

Chemical structures of the indole-based compounds	**IC_{50} for LEDGF/p75-dependent integration (μM)**
 KF110	44.1 ± 3.7
 KF111	32.0 ± 2.5

 KF112	373 ± 72
 KF113	4.5 ± 0.5
 KF114	31.3 ± 0.9
 KF115	59.1 ± 3.4

To dissect the mode action of KF113, I have further analyzed its abilities to inhibit LEDGF/p75-independent IN 3'-processing activity, IN binding to LEDGF/p75 and/or induce aberrant IN multimerization using the above described HTRF-based assays. The results in Figures 9, 10, and 11 and in summary table 6 show that the indole-based KF113 exhibited a multimodal mechanism of action. In particular, this compound inhibited both LEDGF/p75-dependent integration as well

as LEDGF/p75-independent 3'-processing activity of IN (Figure 9 and Table 6). Furthermore, KF113 induced aberrant IN multimerization (Figure 10), which could explain its ability to inhibit LEDGF/p75-independent 3'-processing activity of IN (Table 6). In addition, KF113 was able to inhibit IN-LEDGF/p75 binding (Figure 11). Such a multimodal mechanism of action of this compound is reminiscent to the mechanism of action of quinoline-based compounds, such as BI-B2 (*9*). Indeed, the x-ray crystallography studies conducted by Dr. Feng have revealed that KF113 binds at the IN dimer interface in the principal LEDGF/p75 binding pocket (Figure 12). Therefore, this compound is able to both promote aberrant IN multimerization by bridging between two IN subunits as well as inhibit binding of LEDGF/p75 to the IN dimer interface.

Table 6: IC_{50} values of KF113 compound.

Compound	IC_{50} for LEDGF/p75 dependent Integration	IC50 for LEDGF/p75 independent 3'-proc. activity	IC_{50} for aberrant IN multimerization	IC_{50} for IN-LEDGF/ p75 Binding
KF113	**4.5 ± 0.5**	**3.2 ± 0.5**	**19.7 ± 2.5**	**28.7 ± 3.2**

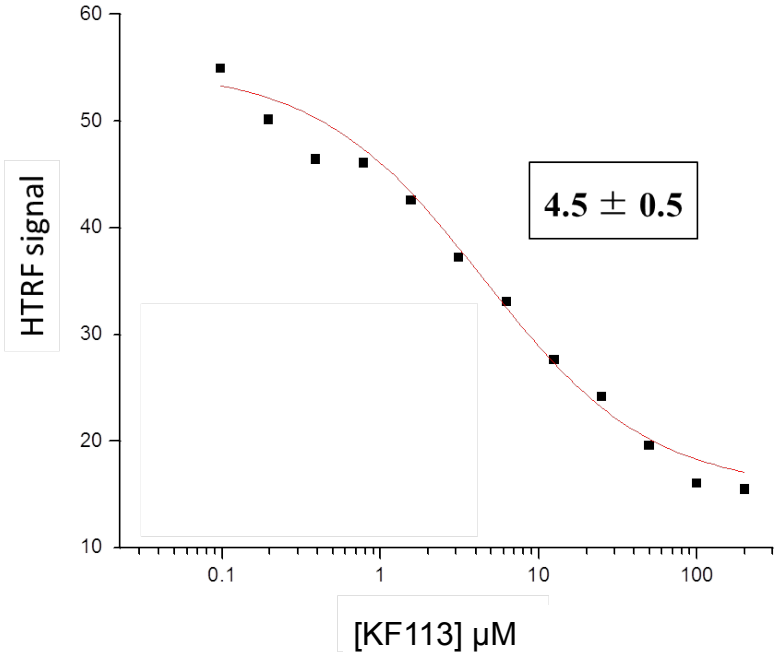


Figure 9. The dose response curve inhibiting LEDGF/p75-dependent integration by KF113.

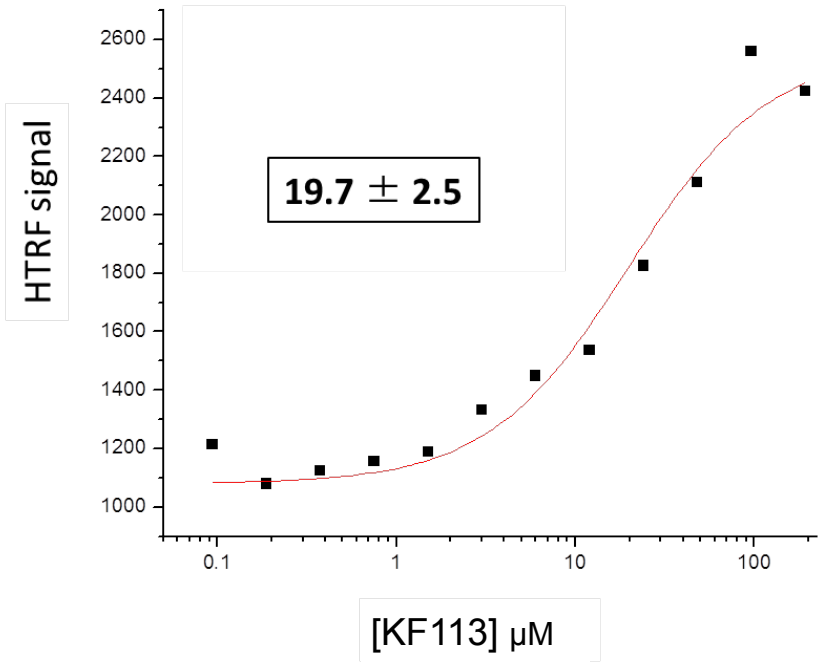


Figure 10. The dose response curve for KF113 promoting aberrant multimerization.

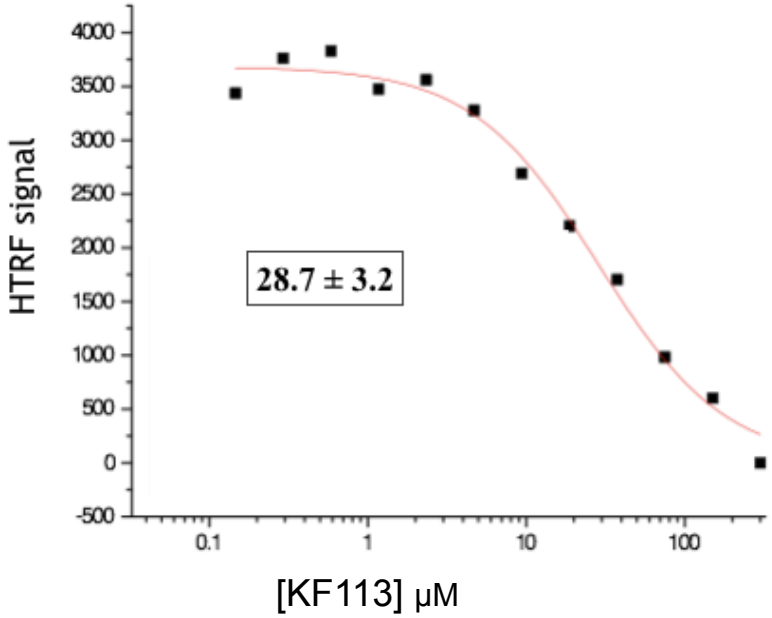


Figure 11. The dose response curve for KF113 inhibiting IN binding to LEDGF/p75.

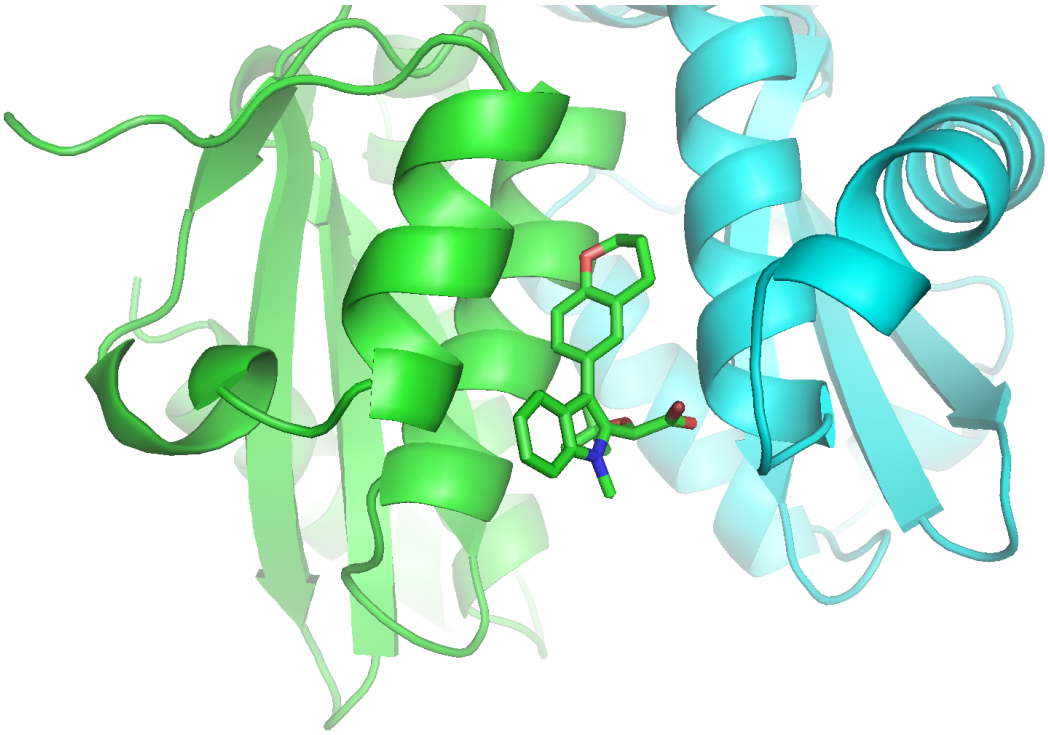


Figure 12. Crystal structure of KF113 bound to the catalytic core domain of IN (determined by Dr. Feng).

Indole-based compounds are potent with respect to the A128T IN mutant that confers resistance to quinoline-based ALLINIs.

I have next assayed activities of KF113 with respect A128T IN. It has previously been shown that A128T IN exhibits remarkable (~1000-fold) resistance to archetypal quinolone-based BI-B2 (*9*). In sharp contrast, KF113 inhibited both wild type and the A128T mutant INs with similar IC_{50} values (Figures 13 and 14). Therefore, while indole-based KF113 binds to the same pocket as quinolone-based ALLINIs, the structural difference between these two classes of compounds allows the indole-based KF113 to retain its activity with respect to A128T IN.

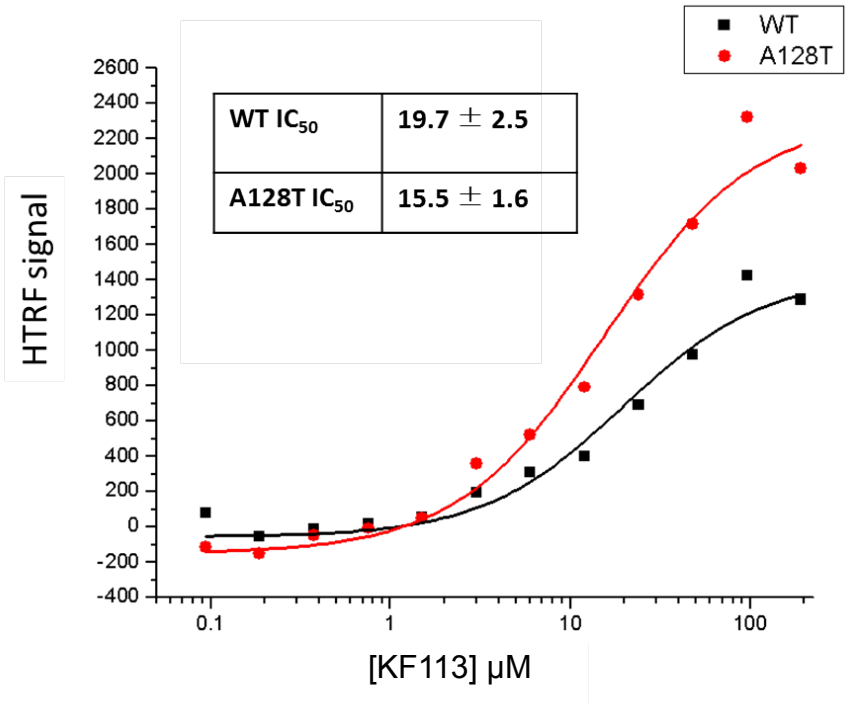


Figure 13. Dose response curves for KF113 promoting aberrant multimerization of WT (black squares) and A128T (red circles) INs.

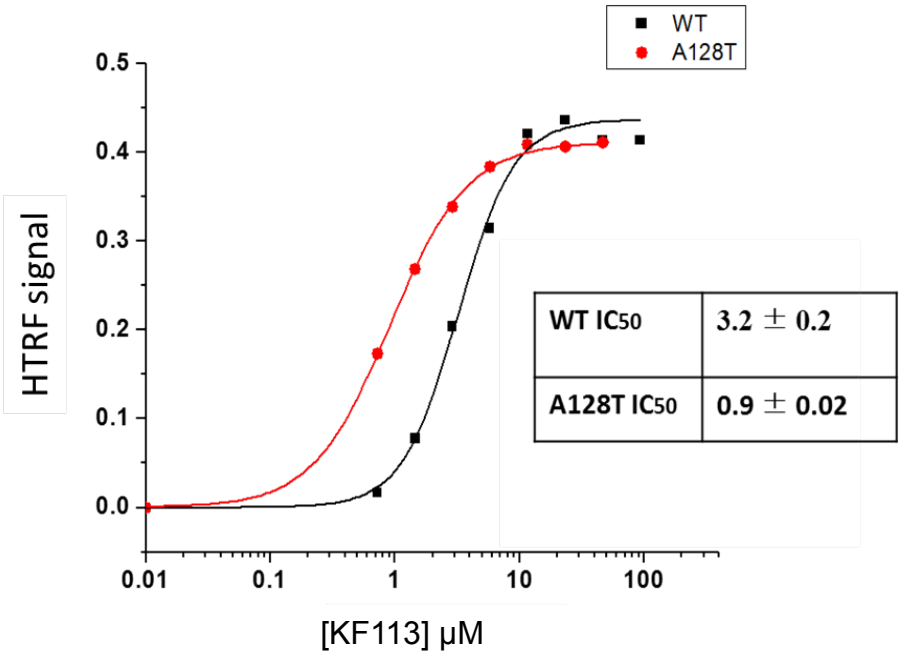


Figure 14. Dose response curves for KF113 inhibiting 3'-processing activity of WT (black squares) and A128T (red circles) INs.

Discussion

The HTRF-based assays are instrumental for dissecting the mode of action of new analogues of ALLINIs. Current quinolone-based ALLINIs, such as archetypal BI-B2, allosterically cause both aberrant IN multimerization and inhibition of IN-LEDGF/p75 binding, and have shown to be promising candidates for development as novel integrase-inhibiting antiretroviral therapy. Yet various mutations, including the A128T mutation, in the integrase catalytic core domain has led to resistance of IN to these compounds. Therefore, we have been investigating a new class of indole-based compounds. It was discovered *in silico* that these analogues would maintain a binding mode similar to BI-B2, but also orient the indole-based structures in a way that they could still work effectively against the A128T mutation. These initial findings prompted Dr. Fuchs to synthesize the indole-based compounds by modifying known structures of quinoline-based ALLINIs. I initially examined six indole-based derivatives using the LEDGF/p75 dependent integration assay. The compound called KF113 was the most active with an IC_{50} value of 4.5 μM. Therefore, I selected this compound for further analysis and found that KF113 induces IN multimerization with an IC_{50} value of 19.7 μM and inhibits IN-LEDGF/p75 binding with an IC_{50} value of 28.7 μM, suggesting that similarly to the quinoline-based compounds, indole-based inhibitors exhibit a multimodal mechanism of action. However, unlike quinoline-based BI-B2, KF113 remained active when tested with the mutant IN A128T. Therefore, the indole-based compounds present promising leads for development of new types of allosteric IN inhibitors.

Citations

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